



## RESEARCH ARTICLE

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# NaCl protects against Cd and Cu-induced toxicity in the halophyte *Atriplex halimus*

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## Abstract

The objective of the present work was to evaluate the extent of Cd- and Cu-induced oxidative stress and the antioxidant response triggered in the halophyte species *Atriplex halimus* after metallic trace elements exposure. Plants were treated for one month with Cd<sup>2+</sup> or Cu<sup>2+</sup> (400 µM) in the absence or presence of 200 mM NaCl in the irrigation solution. The interaction between salinity and heavy metal stress was analyzed in relation to plant growth, tissue ion contents (Na<sup>+</sup>, K<sup>+</sup> and Mg<sup>2+</sup>), oxidative damage and antioxidative metabolism. Data indicate that shoot and root weight significantly decreased as a consequence of Cd<sup>2+</sup>- or Cu<sup>2+</sup>-induced stress. Metallic stress leads to unbalanced nutrient uptake by reducing the translocation of K<sup>+</sup> and Mg<sup>2+</sup> from the root to the shoot. The levels of malondialdehyde increased in root tissue when Cd, and especially Cu, were added to the irrigation solution, indicating that oxidative damage occurred. Results showed that NaCl gave a partial protection against Cd and Cu induced toxicity, although these contaminants had distinct influence on plant physiology. It can be concluded that salinity drastically modified heavy metal absorption and improved plant growth. Salinity also decreased oxidative damage, but differently in plants exposed to Cd or Cu stress.

**Additional key words:** *Atriplex halimus*; oxidative damage; trace element toxicity; salinity

**Abbreviations used:** APX (ascorbate peroxidase); CAT (catalase); DTNB (dinitrobenzoic acid); FW (fresh weight); GPX (guaiacol peroxidase); GSH (reduced glutathione); GSSG (oxidised glutathione); MDA (malondialdehyde); PC (phytochelatin); ROS (reactive oxygen species); SOD (superoxide dismutase); tGSH (total GSH)

**Authors' contributions:** Cation determination assays: IB. Coordinated the obtention and culture of the plants and was responsible for conducting different experiments and the evaluation of plants: NS. Responsible of the study of the antioxidative system, evaluating the antioxidant enzymatic activities as well as MDA; he also was in charge of glutathione and phytochelatin content determination: AGC. Coordination of the works and preparing the manuscript: RMPC.

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## Introduction

Toxic trace elements are bioavailable to plants through their uptake from soil and water. Among pollutants, heavy metals such as cadmium (Cd), copper (Cu), lead and mercury are of particular concern as they are non-biodegradable and strongly phytotoxic. Furthermore, at high concentration, they not only limit plant productivity but also threaten human health (López-Climent *et al.*, 2011).

Based on their ability to cope with heavy metals in the medium where they grow, plants can be divided

into three groups: i) indicator plants, sensitive to heavy metals; ii) excluder plants, being able to tolerate high heavy metal concentrations in the soil by preventing its accumulation the cytosol, and iii) hyperaccumulator plants, that tolerate high concentrations of specific trace elements in the medium, with an active uptake and specific accumulation of those elements (reviewed in Leitenmaier & Küpper, 2013).

Many arid and semi-arid regions of the world, that combine high soil salinity with aridity, have the additional problem that lands are even more degraded, consequence of the human activities such as mining,

agricultural activities, etc. In these particular soils it is important to consider all these stressful conditions (drought, salinity, heavy metal contamination) together, since isolating one adverse condition from the others is not possible. Under these particular circumstances, halophytes have been proposed as candidates for the removal of trace elements from the soil (Manousaki & Kalogerakis, 2009).

There are two main reasons to study  $\text{Cd}^{2+}$  and/or  $\text{Cu}^{2+}$  hyperaccumulator plants. First, there is an interest in acquiring basic knowledge on the mechanisms underlying environmental adaptation and cell detoxification of those trace metals. Second, the study of  $\text{Cd}^{2+}/\text{Cu}^{2+}$  hyperaccumulators can provide new tools to design genetically-modified plants useful for phytoremediation or phytoextraction (Clemens *et al.*, 2013).

Cu is an essential micronutrient for a range of plant physiological processes via the action of  $\text{Cu}^{2+}$ -dependent enzymes. On the contrary, Cd is a non-essential heavy metal ion that causes disturbances in plant growth, photosynthesis, ion and water transport, as well as decreases in plant enzyme activities through reactions between the  $\text{Cd}^{2+}$  and thiol groups. When reaching a certain concentration in plant tissue, both metallic trace elements are involved in the generation of reactive oxygen species (ROS), which react with lipids, proteins, photosynthetic pigments, and nucleic acids, causing lipid peroxidation, membrane damage, metabolite degradation, inactivation of enzymes, and even cell death (Nagarani *et al.*, 2012). The increase of lipid peroxidation products such as malondialdehyde (MDA) as a consequence of the oxidative stress has been considered as an indicator of free radical production and tissue damage. Actually, lipid peroxidation alters membrane properties such as fluidity and permeability and thereby its functions (da Silva *et al.*, 2010).

Plants have developed different mechanisms to control the uptake of both essential and nonessential heavy metals. These mechanisms include the chelation and sequestration of ions by specific ligands to maintain them at low levels in the cytosol (Sbartai *et al.*, 2012). Chelation of trace elements is a ubiquitous detoxification strategy described in a wide range of plants (Yadav, 2010). The main group of substances acting as metal chelators in plants are phytochelatins (PCs), which are metal-binding peptides that also play similar detoxification roles in other eukaryotes, including fungi and microalgae (Hirata *et al.*, 2005). PCs, with a general structure of  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$  ( $n=2\text{--}11$ ), are rapidly synthesized by the cytoplasmic enzyme PC synthase in response to intoxication with heavy metals. Genetic studies have confirmed that glutathione (GSH) is pivotal for PC synthesis as GSH-deficient mutants of *Schizosaccharomyces pombe* and *Arabidopsis* sp are

PC-deficient and hypersensitive to Cd (Lee *et al.*, 2003). PCs production in plants is stimulated by the presence of heavy metals. PCs are metal-binding peptides and work by mobilizing heavy metal compounds in the cytosol and then sequestering PC-metal complexes in the vacuoles of plant cells. The absorbed heavy metal compounds are biochemically bonded by PCs in inert forms where they are eventually compartmentalized (Watanabe, 1997), so PCs could play a crucial role in phytodegradation and phytotransformation.

GSH, a non-enzymatic antioxidant, is a low-molecular-weight thiol involved in various metabolic processes that acts as an important plant defense molecule against environmental stresses, including trace elements (Hossain *et al.*, 2011). It is one of the major antioxidant and redox buffers in plants, abundantly found in all cell compartments. GSH takes part in the control of  $\text{H}_2\text{O}_2$  levels through the ascorbate-GSH cycle and also directly as a free-radical scavenger (Foyer & Noctor, 2005). Additionally, enzymatic activities greatly contribute to cope with the environmentally-induced oxidative stress and their activities have been used to evaluate stress responses in plants (Arbona *et al.*, 2008). Antioxidant enzymes interrupt the cascades of uncontrolled oxidation by removing, neutralizing or scavenging oxy-radicals and their intermediates (Foyer & Noctor, 2005). Superoxide dismutase (SOD) acts as the first line defense against ROS, dismuting  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  which is still toxic and should be eliminated in subsequent reactions, through the action of catalase (CAT), other peroxidases and by the ascorbate-glutathione cycle. The equilibrium of SOD, ascorbate peroxidase (APX) and CAT activities is essential in order to determine the steady-state level of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ . For instance, when CAT activity is reduced in plants, other ROS-scavenging enzymes such as APX and guaiacol peroxidase (GPX) are up-regulated (Vandenabeele *et al.*, 2004).

*Atriplex halimus* L. is a perennial, halophytic and nitrophilous shrub that generally forms part of the halophytic communities on saline soils and it represents one of the best options for the reclamation of degraded agricultural land in arid and semi-arid zones (reviewed in Walker *et al.*, 2014). This species has been planted in the Mediterranean arid zone for livestock feed. In fact, the planted area is estimated at around 100,000 ha in Algeria, the Arabian Peninsula, Egypt, Iraq, Israel, Jordan, Libya, Morocco, Spain and Tunisia (Walker *et al.*, 2014). It has been previously reported that the addition of NaCl to the irrigation solution did not affect plant growth or chlorophyll concentration in *A. halimus*. On the contrary, there was a significant stimulation of its growth, which confirms the halophytic character of this species. Furthermore, a high concentration of NaCl

reverted some of the negative effects that trace element intoxication exerted on growth parameters of these plants (Bankaji *et al.*, 2014).

Taking all this in consideration, the present study was designed to investigate (i) the extent of Cd<sup>2+</sup>- and Cu<sup>2+</sup>-induced oxidative stress in *A. halimus* plants, (ii) differences in the plant antioxidant responses to each metal, and (iii) the effect of Cd<sup>2+</sup> and Cu<sup>2+</sup> under salt stress conditions. These objectives were accomplished by determining lipid peroxidation, and also examining changes in antioxidant enzyme activities such as CAT and APX together with glutathione and PCs contents in different tissues of *A. halimus* plants exposed to toxic levels of Cu<sup>2+</sup> and Cd<sup>2+</sup> alone or in combination with high NaCl concentration in the irrigation solution.

## Material and methods

### Plant material and culture

Plants of *Atriplex halimus* (Chenopodiaceae) used in the experiments were obtained by cutting propagation: 5-cm long fragments of leafy stems were taken from mother plants, rinsed abundantly with distilled water, and placed for rooting in plastic pots (3 dm<sup>3</sup>) containing a mixture of perlite/gravel (2:1) as a substrate. In these conditions, rooting occurred one month after planting (Bankaji *et al.*, 2014). Young rooted cuttings were irrigated three times a week with 200 mL modified Hewitt nutritive solution (Hewitt, 1966) as described in Bankaji *et al.* (2014).

### Experimental design

Acclimated rooted cuttings, homogeneous in size and looking were randomly distributed in control and five stress treatments. Briefly, 20 control plants and 20 plants per stress treatment were used. All plants were regularly irrigated (three times a week) with the same nutritive solution described above but for stressed plants it was supplemented with a) 400 µM CdSO<sub>4</sub>; b) 400 µM CuSO<sub>4</sub>; c) 200 mM NaCl; d) 400 µM CdSO<sub>4</sub> and 200 mM NaCl or e) 400 µM CuSO<sub>4</sub> and 200 mM NaCl. During the experimental period, plants were maintained in a greenhouse under natural photoperiod, average temperatures (night/day) of 12/25°C and relative humidity between 60-90%. According to preliminary experiments, concentration of 400 µM for CdSO<sub>4</sub> and CuSO<sub>4</sub> proved to be higher enough to induce negative effects on plant performance. It was expected that the negative effects could be reverted by the addi-

tion of NaCl to the irrigation solution. Treatments were maintained for one month.

At harvest, four weeks after the onset of treatments, plants of each treatment were randomly divided into groups of five plants for further analysis. In one group, leaves were separated from roots, washed with distilled water, frozen in liquid nitrogen and kept at -80 °C until GSH, PC and MDA analyses. Five different plants were processed similarly and used for the enzymatic activities assays. The remaining plants, which were used for the cation assays, were divided into leaves and roots, rinsed three times in cold water and blotted with filter paper. Treated roots were dipped in a cold solution of CaCl<sub>2</sub> during 5 min (Stolt *et al.*, 2003) to eliminate trace elements adsorbed at the root surface and then rinsed three times with cold distilled water. The fresh weight was immediately determined in roots and shoots before separating the leaves. The same experimental design was repeated twice with identical results.

### Cation assays

Fresh plant material was digested by mixture of HNO<sub>3</sub>:HClO<sub>4</sub> acids (4:1) (Barthwal *et al.*, 2008). Mineralization was conducted gradually from 100 to 350°C for 2 hours. After that, the samples were taken into 50 mL of nitric acid 0.5%. Finally, these extracts were filtered and concentrations of Cu<sup>2+</sup>, Cd<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup> and Mg<sup>2+</sup> in plant tissues were determined by atomic absorption spectrometry (Perkin Elmer PinAAcle 900T, USA). The blanks, used to set the zero atomic absorption spectrometer, were similarly processed as described above.

### Antioxidant enzyme activity

For CAT, APX and GPX assays, extraction was performed as follows: 0.4 g of fresh leaf tissue was powdered using liquid nitrogen and extracted, at 4°C, with 2,0 ml of phosphate buffer containing 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.0), 5 mM Na-ascorbate and 0.2 mM EDTA. After filtration through four layers of musceline cloth, the homogenate was centrifuged at 6000 rpm for 15 min and the supernatant recovered for enzyme activities determination using a UV/Visible spectrophotometer (Thermo Spectronic Genesis UV10, USA).

Catalase (H<sub>2</sub>O<sub>2</sub>:H<sub>2</sub>O<sub>2</sub> oxidoreductase, EC1.11.1.6) activity was determined by monitoring the disappearance of H<sub>2</sub>O<sub>2</sub> by measuring the decrease in absorbance at 240 nm (extinction coefficient of 0.036/mM·cm) of a reaction mixture containing 25 mM K- phosphate

buffer (pH 7.0), 10 mM  $\text{H}_2\text{O}_2$  and 2 mL of enzyme extract (Aebi, 1984).

For GPX (Guaiacol:  $\text{H}_2\text{O}_2$  oxidoreductase, EC1.11.1.7), the reaction mixture consisted of 25 mM K-phosphate buffer (pH 7.0), 10 mM  $\text{H}_2\text{O}_2$ , 9 mM guaiacol and 2 mL enzyme extract. The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (extinction coefficient of  $26.6/\text{mM}\cdot\text{cm}$ ) during polymerization of guaiacol (Fielding & Hall, 1978).

APX (L-Ascorbate:  $\text{H}_2\text{O}_2$  oxidoreductase, EC1.11.1.11) was determined according to Nakano & Asada (1981) measuring ascorbate consumption at 290 nm (absorbance coefficient of  $2.8/\text{mM}\cdot\text{cm}$ ). The reaction mixture contained 25 mM K-phosphate buffer (pH 7.0), 0.5 mM ascorbate, 2 mM  $\text{H}_2\text{O}_2$ , 0.1 mM EDTA and 2 mL of enzyme extract.

### Determination of total PC and GSH concentrations

Total non-protein thiol (TNP-SH) compound were extracted and assayed according to de Vos *et al.* (1992). Briefly, 100 mg of fresh weight (FW) plant tissue were rapidly frozen in liquid nitrogen and exhaustively extracted with 2 mL of 5% 5-sulphosalicylic acid with 6.3 mM diethylenetriaminepentaacetic acid (DETA-PAC) using a pre-chilled mortar, pestle and quartz sand. The homogenate was filtered and centrifuged at 8000 rpm for 5 min at  $4^\circ\text{C}$ . The N-P thiols were measured in the supernatant by diluting it with reagent containing 0.5 M K-phosphate buffer (pH 7.5) and 6 mM dinitrobenzoic acid (DTNB). Absorbance was recorded at 412 nm.

The DTNB-oxidised glutathione (GSSG) reductase recycling procedure of Anderson (1985) was used for the determination of both total [GSH (reduced glutathione) + GSSG] and oxidized glutathione. Briefly, 100 mg of fresh tissue was homogenized in 2 mL of 5% (w/v) cold metaphosphoric acid. Homogenate was centrifuged at  $4^\circ\text{C}$  to pellet debris and supernatant neutralized with 0.5 M phosphate buffer (pH 7.5). Total GSH (tGSH) was measured in a reaction mixture containing 125 mM Na-phosphate buffer (pH 7.5) with 6.3 mM Na-EDTA, 6 mM DTNB, 0.3 mM NADPH and the sample. Reaction was initiated by adding pure glutathione reductase (Sigma-Aldrich, Madrid, Spain) to the reaction mixture and monitored by measuring absorbance changes at 412 nm. For measuring GSSG, plant extracts were incubated at  $25^\circ\text{C}$  with 2-vinylpyridine and triethanolamine (Fluka, Madrid, Spain) for 1 h prior the determinations. The assay for GSSG was conducted as described above for tGSH. GSSG and

tGSH contents were estimated by interpolating slope values in a calibration curve performed with pure standards. GSH content was calculated by subtracting GSSG content from the tGSH content. PC production was evaluated as PC-SH levels by subtracting the amount of GSH from the amount of TPN-SH compounds (de Vos *et al.*, 1992).

### MDA concentration

MDA concentration was measured following the procedure described in Hodges *et al.* (1999). Plant material (0.4 g root or leaf tissue) was homogenized in 5 mL of 80% cold ethanol (Panreac, Barcelona, Spain) using a tissue homogenizer (Ultra-Turrax; IKA-Werke, Staufen, Germany). Homogenates were centrifuged at  $4^\circ\text{C}$  to pellet debris and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid (TCA, Panreac) or a mixture of 20% TCA and 0.5% thiobarbituric acid (TBA, Sigma-Aldrich). Both mixtures were allowed to react in a water bath at  $90^\circ\text{C}$  for 1 h. After this time, samples were cooled down in an ice bath and centrifuged. Absorbance at 440, 534 and 600 nm was read in the supernatant against a blank. The MDA concentration in the extracts was calculated as in Arbona & Gomez-Cadenas (2008).

### Statistical analysis

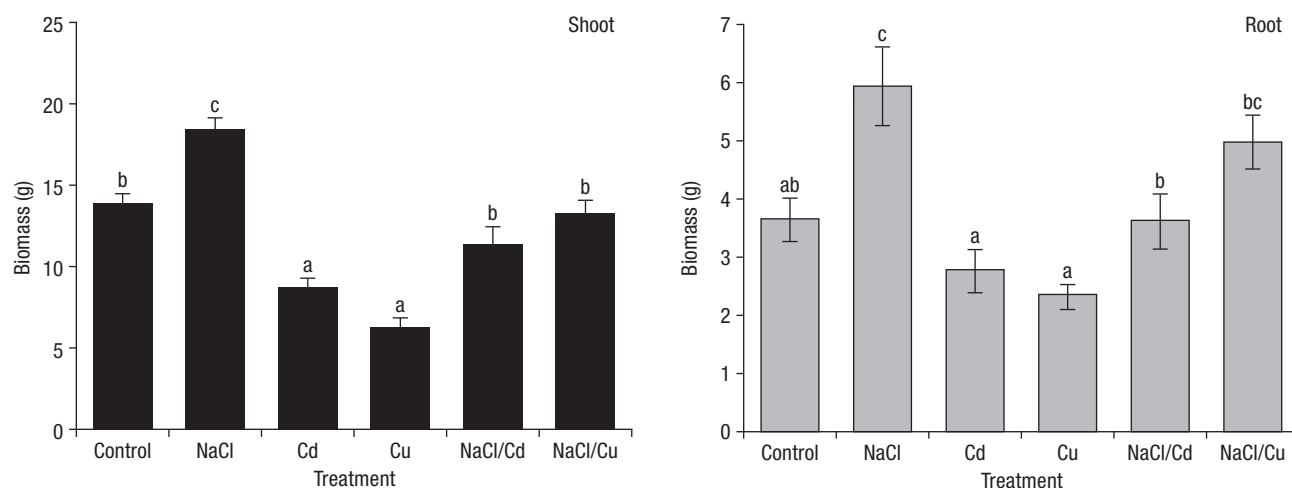
All the data were subjected to analysis of variance (ANOVA) and the significance of the Tukey test was determined at 5% level. Statistical analyses were performed with the Statistica 8 for Windows software.

## Results

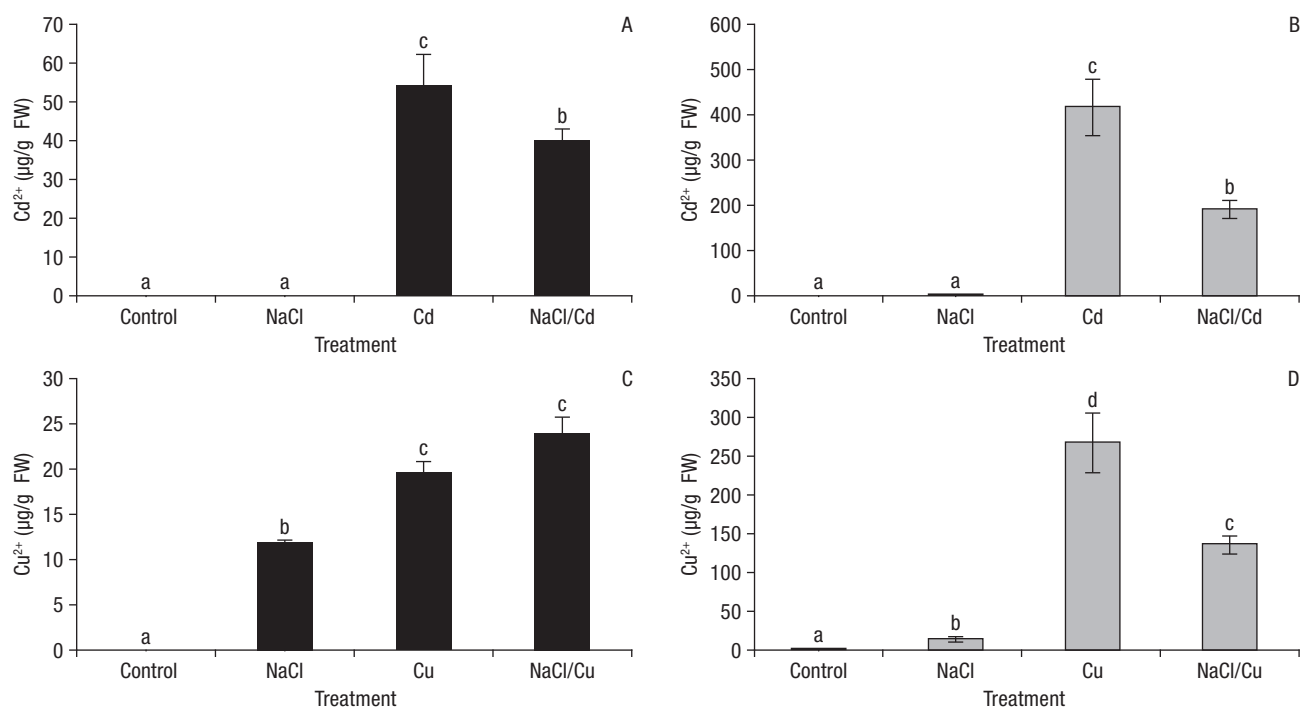
### Plant growth

Biomass production significantly decreased in shoots of *A. halimus* plants cultivated in a medium supplemented with  $\text{Cd}^{2+}$  (Fig. 1). Reductions in FW were detected both in shoots (37.5%) and in roots (24.1%) when  $400\text{ }\mu\text{M}$   $\text{Cd}^{2+}$  was added to the irrigation solution. Similarly, the addition of  $400\text{ }\mu\text{M}$   $\text{Cu}^{2+}$  to the irrigation solution reduced biomass production by 54.6% and 36.4% in shoots and roots, respectively. When the solution was supplemented with NaCl (200 mM) the negative effect of both trace elements was reverted, and FW in shoots and roots reached values even higher to those found in control plants (Fig. 1).





**Figure 1.** Biomass production in shoot and root tissue of *Atriplex halimus* exposed to 400  $\mu\text{M}$   $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values  $\pm$  SE,  $n=10$ . Different letters denote significant differences at  $p \leq 0.05$  on each treatment.



**Figure 2.** Cadmium (A,B) and copper (C,D) concentration in leaf (A,C) and root (B,D) tissue of *Atriplex halimus* exposed to 400  $\mu\text{M}$   $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values  $\pm$  SE,  $n=10$ . Bars marked with the same letter are not significantly different at  $p \leq 0.05$ .

Shoot and root water contents did not show any significant alteration in response to NaCl or heavy metal treatment, showing values between 76.8 and 84.2% (data not shown).

### $\text{Cu}^{2+}$ and $\text{Cd}^{2+}$ concentration

$\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  concentration in leaves and roots of *A. halimus* plants were analyzed at the end of the ex-

periment (after 4 weeks of treatment) and results are shown in Fig. 2.  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  concentrations in control plants were negligible or below the detection limit, both, in root and leaf tissues. When trace elements were added to the irrigation solution,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  accumulation, was, as expected, higher in root than in leaf tissues. The concentration of  $\text{Cd}^{2+}$  was 417.1 and 54.0  $\mu\text{g/g}$  FW in roots and leaves, respectively when plants were irrigated with a solution supplemented with 400  $\mu\text{M}$   $\text{Cd}^{2+}$ . On the other hand,  $\text{Cu}^{2+}$  content was

269.1 and 19.6  $\mu\text{g/g}$  FW in roots and leaves, respectively when 400  $\mu\text{M}$   $\text{Cu}^{2+}$  was added to irrigation solution.

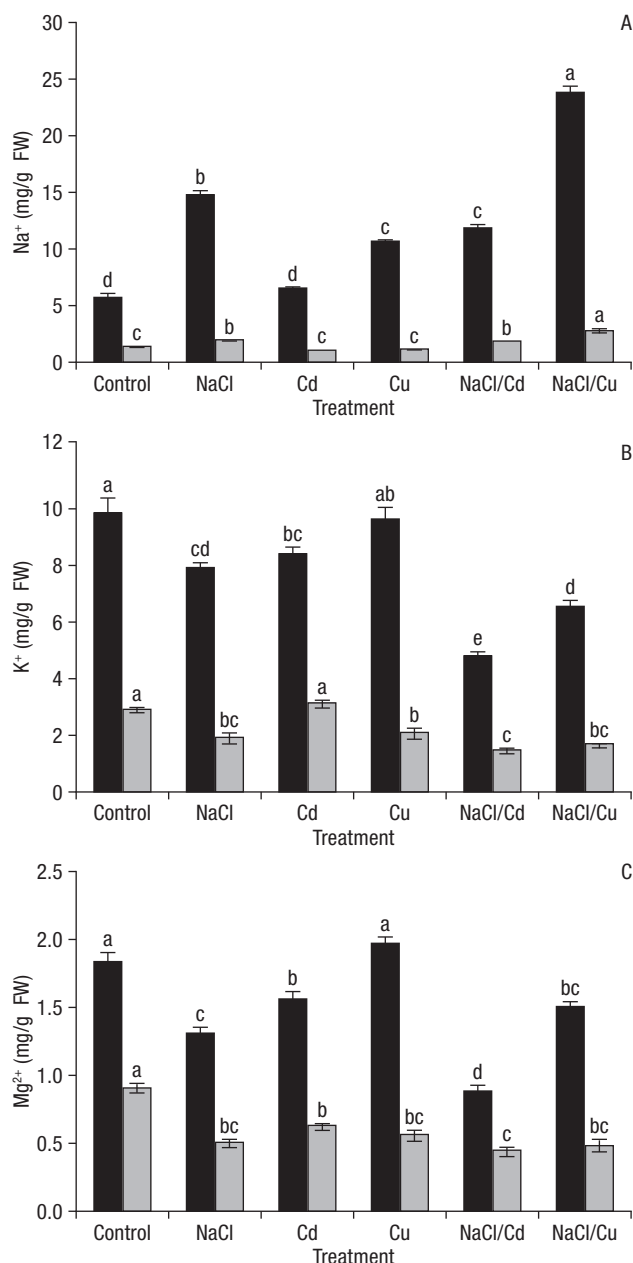
The addition of 200 mM NaCl to the irrigation solution significantly reduced the endogenous  $\text{Cd}^{2+}$  concentrations (Fig. 2A,B). In root tissue, the presence of NaCl induced a reduction of 53.4% whereas in leaf tissue,  $\text{Cd}^{2+}$  content was reduced by a 25.9%. When NaCl was simultaneously added with  $\text{Cu}^{2+}$ , a reduction of 48.7% in  $\text{Cu}^{2+}$  content was recorded in root tissue (Fig. 2D); on the contrary the endogenous  $\text{Cu}^{2+}$  concentration in leaf tissue was not significantly affected by the NaCl supply (Fig. 2C).

### Nutrient concentration

Roots of plants treated with  $\text{Cu}^{2+}$  or  $\text{Cd}^{2+}$  had  $\text{Na}^+$  contents similar to those measured in control ones. When  $\text{Cu}^{2+}$  was incorporated to the irrigation solution together with NaCl, the endogenous root  $\text{Na}^+$  concentration was significantly higher than that recorded in roots of plants irrigated with high concentrations of NaCl (without the trace element). In the case of  $\text{Cd}^{2+}$ , no differences in root  $\text{Na}^+$  concentration were recorded between plants irrigated with a solution supplemented with Cd alone or in combination with NaCl (Fig. 3A). The presence of  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  in the irrigation had different effect on leaf  $\text{Na}^+$  accumulation; while solution supplemented with  $\text{Cd}^{2+}$  did not affect accumulation of  $\text{Na}^+$  in the aerial part, the addition of  $\text{Cu}^{2+}$  caused a significant increase in  $\text{Na}^+$  concentration reaching values 1.8-fold higher than those measured in controls (Fig. 3A).

The presence of NaCl in the medium significantly decreased leaf  $\text{K}^+$  concentration from 9561.2 to 7679.0  $\mu\text{g/g}$  FW. Similarly, a decrease in this parameter was observed in plants treated with  $\text{Cd}^{2+}$  either alone or in combination with NaCl (Fig. 3B). The addition of  $\text{Cu}^{2+}$ , in combination with NaCl, to the irrigation solution also reduced leaf  $\text{K}^+$  concentration, on the contrary, when  $\text{Cu}^{2+}$  was added alone,  $\text{K}^+$  concentration was similar to that measured in leaves of control plants (Fig. 3B).

The addition of NaCl or  $\text{Cd}^{2+}$  to the irrigation solution significantly decreased leaf  $\text{Mg}^{2+}$  concentration (33.4 and 20.4% reduction respectively). When both (NaCl and  $\text{Cd}^{2+}$ ), were supplemented simultaneously, the reduction of this micronutrient content in leaves was more severe (54.6%). On the contrary, the addition of  $\text{Cu}^{2+}$  to the irrigation solution had no effect on this parameter although when this metal was provided simultaneously with NaCl, a significant reduction of leaf  $\text{Mg}^{2+}$  concentration (23.7%) was recorded. Root  $\text{Mg}^{2+}$

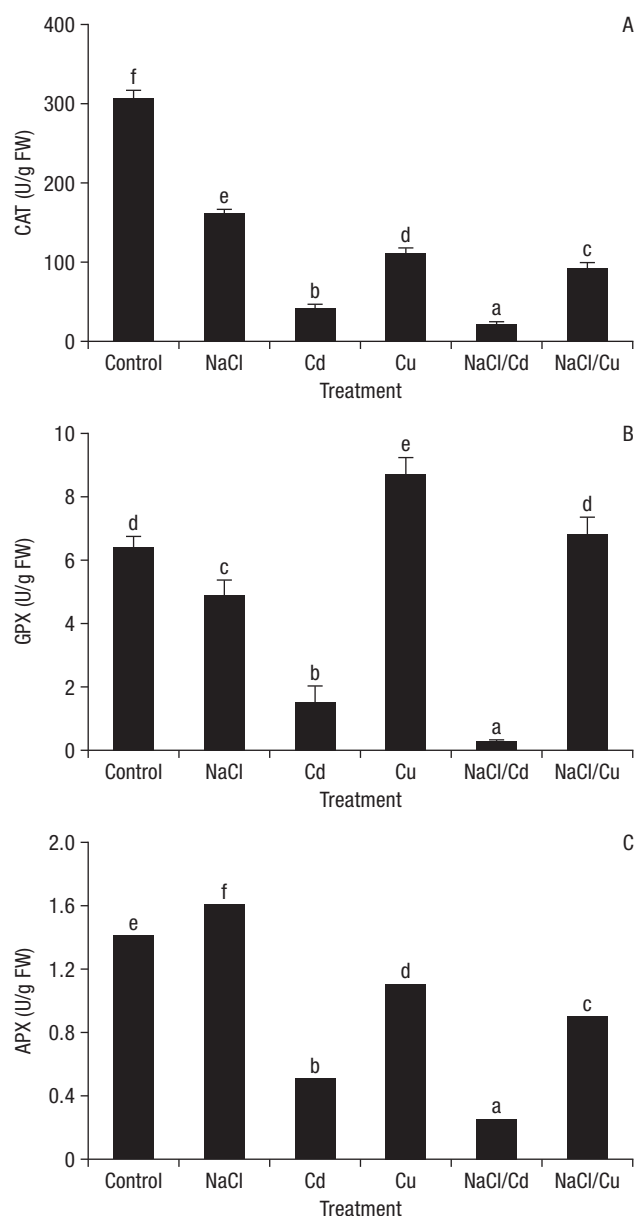


**Figure 3.** Sodium (A), potassium (B) and magnesium (C) concentration in leaves (bars in black) and roots (bars in grey) tissue of *Atriplex halimus* exposed to 400  $\mu\text{M}$   $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values  $\pm$  SE,  $n=10$ . In each plant material, bars marked with the same letter are not significantly different at  $p \leq 0.05$ .

concentration significantly decreased in *A. halimus* under  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  stress, supplemented or not with NaCl (Fig. 3C).

### Antioxidant enzyme activity

The increase in  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  content in plant tissue had significant effects on the activities of antioxidant enzymes (Fig. 4). In leaves, the addition of either  $\text{Cd}^{2+}$

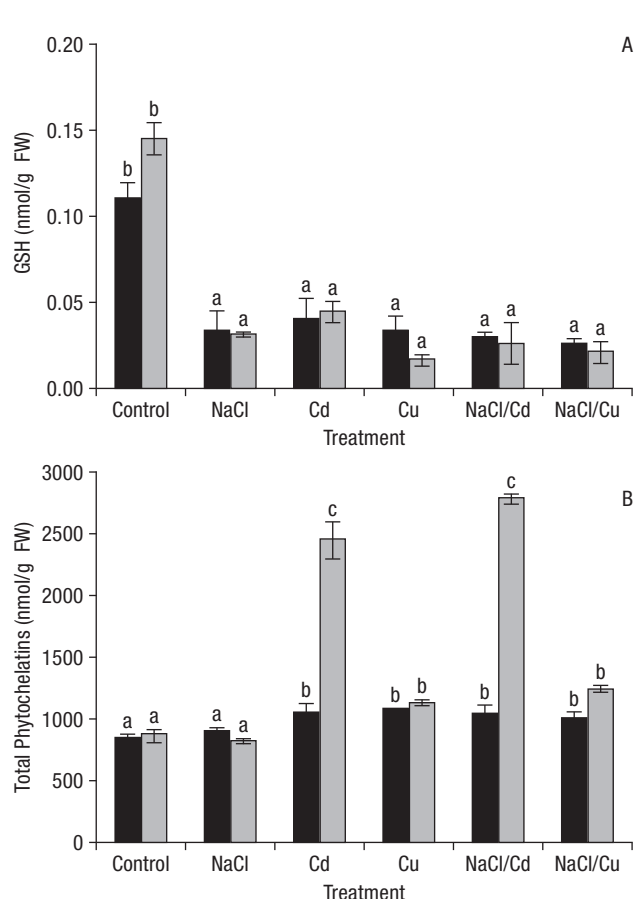


**Figure 4.** Enzymatic activity in leaves of *Atriplex halimus* exposed to 400  $\mu\text{M}$   $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  in the absence or presence of 200 mM NaCl in nutrient solution. Data are mean values  $\pm$  SE,  $n=10$ . Bars marked with the same letter are not significantly different at  $p=0.05$ . Catalase (A), guaiacol peroxidase (B) and ascorbate peroxidase (C).

or  $\text{Cu}^{2+}$  to the irrigation solution induced a significant decrease in CAT activity (86.3% and 64.2% after  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  treatments, respectively).

GPX activity decreased 75.8% under  $\text{Cd}^{2+}$  stress (Fig. 4B). In the case of APX activity it was recorded a reduction of 64.3% and 21.4% after  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  treatments respectively (Fig. 4C).

All studied enzymatic activities significantly decreased when  $\text{Cd}^{2+}$  and NaCl were added simultaneously to the irrigation solution, in comparison to those measured when  $\text{Cd}^{2+}$  or NaCl were added alone.



**Figure 5.** Reduced glutathione (A) and phytochelatins (B) content in leaves (bars in black) and roots (bars in grey) of *Atriplex halimus* under Cd or Cu stress. Data are mean values  $\pm$  SE,  $n=10$ . In each plant material, bars marked with the same letter are not significantly different at  $p \leq 0.05$ .

Similar trend was observed for  $\text{Cu}^{2+}$  and NaCl treatment in the case of CAT and APX. Differently, GPX activity in NaCl plus  $\text{Cu}^{2+}$  or  $\text{Cu}^{2+}$  treated plants was significantly higher than in NaCl treated plants (Fig. 4B).

### Glutathione and phytochelatin contents

As it is shown in Fig. 5, GSH levels experienced significant decrease in both roots and leaves of *A. halimus* when the irrigation solution was supplemented with  $\text{Cd}^{2+}$  (66.6% and 63.6% reduction, respectively). Similar results were observed in plants treated with  $\text{Cu}^{2+}$  (86.7% and 72.7% reduction in roots and leaves, respectively). When NaCl and  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  were simultaneously applied to the irrigation solution, there were no significant differences in GSH content both in leaf and root tissues in comparison with the values recorded when NaCl or the trace element were added to the solution in isolation.

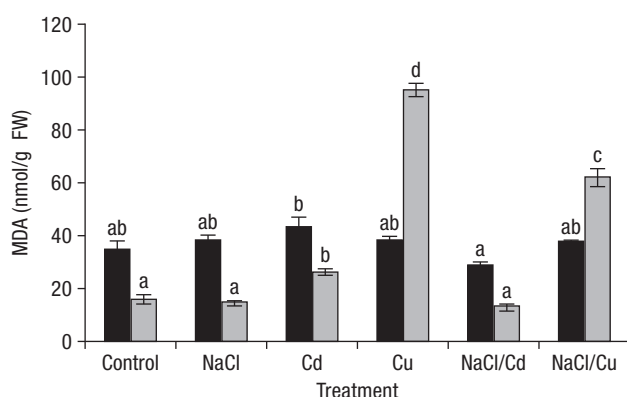
The decrease of GSH levels was accompanied by increases in the synthesis of phytochelatin (Fig. 5B) mainly in roots exposed to high exogenous  $\text{Cd}^{2+}$  concentrations. In this condition PCs levels were 2.8-fold higher than to controls. Phytochelatin accumulation also increased in roots and leaves when the irrigation solution was supplemented with  $\text{Cu}^{2+}$  although to a lesser extent. The addition of NaCl to the irrigation solution containing either  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  did not alter the PCs levels determined when the solution was supplemented only with those metallic trace elements (Fig. 5B).

## Lipid peroxidation

Lipid peroxidation was measured and expressed in terms of MDA. The addition of 200 mM NaCl to the irrigation solution did not have any effect on MDA content, neither in root or leaf tissues (Fig. 6). Plants irrigated with this high saline solution exhibited MDA levels similar to those recorded in control plants. The concentration of this metabolite increased in root tissue when plants were irrigated with a solution containing  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  reaching 1.6- and 5.9-fold increase, respectively, compared to controls. Although the addition of NaCl alone to the irrigation solution did not alter basal levels of MDA in root tissue, when  $\text{Cu}^{2+}$  was simultaneously added, significantly higher values of MDA were recorded. In leaf tissue, MDA concentration was similar among treatments (Fig. 6).

## Discussion

*Atriplex halimus* L. is a perennial halophytic shrub which grows throughout the Mediterranean basin in areas



**Figure 6.** Malondialdehyde content in leaves (bars in black) and roots (bars in grey) of *Atriplex halimus* under Cd or Cu stress. Data are mean values  $\pm$  SE,  $n=10$ . In each plant material, bars marked with the same letter are not significantly different at  $p \leq 0.05$ .

of low annual rainfall and high potential evapotranspiration, many of which can be classified as arid or semi-arid.

Although there are some works that, through the study of different physiological parameters, attempted to address the responses of different halophyte species exposed to abiotic stress caused by the presence of heavy metals in the soil, to our knowledge, there is no information on how, in these conditions, elevated salinity in the substrate may affect plant performance.

This could have practical implications in phytoremediation, since in many arid and semi-arid regions of the world relatively high soil salinity is present in heavy metal contaminated soils. Taking into consideration the results described in this work, it could be also considered the potential use of this species as an alternative method for soil desalination, where salt could be removed from the soil instead of being washed downwards by water.

It has been described that high concentrations of metallic trace elements in the soil may cause plant growth impairment. The inhibition of shoot growth could be due to a decrease in photosynthesis, a disturbance in the mineral nutrition and water balance, and/or to changes in the hormonal status. Results described in this work indicate that shoot and root-biomass of *A. halimus* plants decreased significantly as a consequence of  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$ -induced stress. However, the negative effect of these metallic trace elements was reverted when NaCl was added to the irrigation solution. This finding is in concordance with those described in *Solanum nigrum*, which has been proposed as a Cd-accumulator species (Xu *et al.*, 2010). An important mechanism of plant adaptation to environmental adverse conditions is the structural alteration of the root system. Supplementation of the irrigation solution with NaCl would increase the area and thickness of the leaves and the number of lateral roots, causing increases in the efficiency of photosynthesis and the uptake of water and nutrition in the root system, thereby increasing the biomass of the plants under  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  stress conditions.

Indeed, our results are in agreement with those reported by Lefèvre *et al.* (2009) and Han *et al.* (2012) in the halophyte species *A. halimus* and *Kosteletzkya virginica*. According to these authors, a beneficial impact of exogenous NaCl on plant response to  $\text{Cd}^{2+}$  may be, at least partly, linked to NaCl-induced growth stimulation classically reported for halophyte at moderate salinities, which therefore leads to a dilution effect.

According to Zu *et al.* (2005), hyperaccumulators are plants that can accumulate more than 10 to 500 times concentration of element trace in their aerial parts than a normal plant does. Taking this into consideration, the results described in this and previous works



(Bankaji *et al.*, 2014) suggest that *A. halimus* is a  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  hyperaccumulator species.

*A. halimus* is used for soil stabilization against erosive forces since its deep root system reduces soil compaction and improves aeration (Clemente *et al.*, 2012). In this species, the addition of NaCl to the irrigation solution significantly reduced the endogenous  $\text{Cd}^{2+}$  concentration in different tissues (Fig. 2). On the contrary, salinity induced an increase of leaf  $\text{Cu}^{2+}$  concentration and a significant reduction in  $\text{Cu}^{2+}$  content in root tissues. These results are in clear contrast to those reported by Lopez-Chuken & Young (2005) showing that salinity induces an increase in  $\text{Cd}^{2+}$  levels rather than a decrease in halophyte species. According to these studies, this increase is the consequence of an impact of salinity on the soil and not on the plant. Addition of chloride salts to soil induces an increase in  $\text{Cd}^{2+}$  free ion activity following cation exchange with the salt cation (Lefèvre *et al.*, 2009). This effect, however, could be ruled out in the present work since the experiments here described were performed using a chemically inert substrate, where the formation of such complexes do not occur, and then, trace element absorption is not facilitated. It could be interesting to confirm this idea by experiments using soil as substrate, which take into consideration the bioavailability of some other ions presents in the substrate.

Meanwhile, it might be possible that in the conditions described in this work, salinity has an effect reducing the capacity of roots for ion uptake or even that  $\text{Na}^+$  competitively inhibits  $\text{Cd}^{2+}$  uptake (Bankaji *et al.*, 2014).

Halophytes are characterized by their ability to replace  $\text{K}^+$  by  $\text{Na}^+$  for specific functions, as for maintaining vacuolar osmotic equilibrium (Gattward *et al.*, 2012). As it has been reported for other halophytes (Pan *et al.*, 2011), the addition of NaCl to the irrigation solution significantly decreased shoot  $\text{K}^+$  content as the absorption of this ion was reduced. The effect of the addition of  $\text{Cd}^{2+}$  or  $\text{Cu}^{2+}$  on  $\text{K}^+$  content differs since  $\text{Cd}^{2+}$  significantly reduced shoot  $\text{K}^+$  concentration while  $\text{Cu}^{2+}$  did not affect this parameter, suggesting that  $\text{Cd}^{2+}$  impaired  $\text{K}^+$  uptake in the roots or translocation to the aerial tissues.

The addition of  $\text{Cu}^{2+}$  or  $\text{Cd}^{2+}$  to the medium was accompanied by an alteration of ionic equilibrium within the shoot and root tissues, and again Cd y Cu differed in this respect.  $\text{Cd}^{2+}$  inhibited  $\text{Mg}^{2+}$  (an essential mineral nutrient for photosynthetic metabolism and for phloem loading) uptake and translocation. On the contrary, after  $\text{Cu}^{2+}$  treatment, shoots exhibited similar  $\text{Mg}^{2+}$  values than those recorded in controls. Results pointed out in this work demonstrate that heavy metals present in solution have an impact on  $\text{Na}^+$  absorption, and once more, Cd and Cu had different effect.  $\text{Cd}^{2+}$  did not cause  $\text{Na}^+$  accumulation in shoots,

but an increase in shoot  $\text{Na}^+$  concentration was observed after  $\text{Cu}^{2+}$  exposure. In terms of  $\text{Na}^+$  shoot concentration, a synergistic effect of both,  $\text{Cu}^{2+}$  and NaCl was observed. The specific increase in  $\text{Na}^+$  concentration observed in root and leaf tissues of plants simultaneously exposed to NaCl and  $\text{Cu}^{2+}$  suggests that  $\text{Na}^+$  could play a specific role in response to abiotic stress. Similarly, in other C4 species an increase in  $\text{Na}^+$  endogenous content has been found after plant exposure to different abiotic stress conditions, this could be because Na is needed for an efficient phosphoenolpyruvate regeneration (Lutts *et al.*, 2004).

$\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  induce the formation of harmful ROS, which causes irreversible damage to macromolecules such as lipid and proteins. Lipid peroxidation is widely used as marker of stress-induced damage. In this study, MDA content, a by-product of lipid peroxidation (da Silva *et al.*, 2010) was used as an indirect measurement. Our results indicate that MDA levels increased in root tissue when  $\text{Cd}^{2+}$ , and especially  $\text{Cu}^{2+}$ , were added to the irrigation solution, indicating that oxidative damage occurred. When NaCl was added to the irrigation solution simultaneously with Cd or Cu, MDA levels were reduced, which suggests protective role of NaCl against the stress caused by heavy metals. According to Mourato *et al.* (2012), it can be indicated that the toxic effect of trace elements is probably exerted through free radical generation.

ROS have to be detoxified for the protection of plant cells against the toxic effect of these species. The differences in subcellular localization and biochemical properties of antioxidant enzymes and the distinct responses in gene expression, in addition to the presence of non-enzymatic mechanisms, result in a versatile and flexible antioxidant system able to control the optimum ROS levels (Vranova *et al.*, 2002). However, the antioxidant response of plants under heavy metal stress depends greatly on the genotype, metal species and stress treatment duration. It has been reported that exposure of pea (*Pisum sativum* L.) plants to cadmium altered enzymatic and non-enzymatic antioxidant defenses (Romero *et al.*, 2007). On the contrary, in coffee plants, the activity of APX increased at lower cadmium concentration but it was not detectable in cells subjected to a higher cadmium level (Gomes-Junior *et al.*, 2006). In rice, the transcript levels of all APX encoding genes significantly increased after eight hours of exposure to 20 ppm aluminum (Rosa *et al.*, 2010). In pea plants, gene expression of the cytosolic APX isoform increased or declined depending on the aluminum concentration; however, APX enzyme activity did not show any significant change in response to aluminum (Panda & Matsumoto, 2010). In cell suspensions of *Coffea arabica* L., treatment with nickel induced a rapid in-

crease in APX activity, with differences in the degree of induction associated to the heavy metal concentration (Gomes-Junior *et al.*, 2006). Our results show that the addition of  $\text{Cd}^{2+}$  to the irrigation solution induced a significant decrease in different enzymatic activities (CAT, GPX and APX) in *A. halimus*. This decrease could result from the damage caused by metal ion-induced oxygen species (Kachout *et al.*, 2009). When NaCl was simultaneously added, the reduction was more drastic. Similarly,  $\text{Cu}^{2+}$  induced a decrease in CAT and APX activities but increased GPX activity. It is known that while Cu acts as catalysts in the formation of reactive oxygen species (Fenton reaction) and causes GSH depletion, Cd does not appear to generate free radicals, but it increases lipid peroxidation and decrease GSH content, which results indirectly in active oxygen species production. A previous report has shown that these oxygen species cause oxidative damage to antioxidant enzymes diminishing their activities (Kachout *et al.*, 2009). All these findings emphasize the importance and complexity of the interactions of the antioxidant enzymatic activities with other antioxidants in fine tuning of plant antioxidant metabolism.

GSH is a key metabolite in PC synthesis and therefore essential for toxic metal neutralization (López-Climent *et al.*, 2014), its concentration after Cd or Cu exposure, alone or together with NaCl, decreased in *A. halimus* together with an increased synthesis of PCs, mainly in roots of  $\text{Cd}^{2+}$  treated plants. This depletion may be attributed to the involvement of GSH in the synthesis of PCs induced by  $\text{Cd}^{2+}$  and the formation of Cd-GSH complexes (Mendoza & Moreno, 2006).

As a protection from heavy metal intoxication, plants have developed mechanisms to immediately inactivate metal ions entering the cytosol (López-Climent *et al.*, 2014). PCs are rapidly induced *in vivo* by a wide range of heavy metal ions, including  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$ . Data presented herein demonstrate that  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  stress caused PC accumulation in *A. halimus* that can be considered as hyperaccumulator species.

As conclusion, salinity drastically modified heavy metal accumulation and improved plant growth. High levels of NaCl also decreased oxidative damage, but differently in plants exposed to Cd or Cu stress. Under high concentrations of  $\text{Cd}^{2+}$ , oxidative damage was reduced by NaCl treatment in both, root and shoots tissue whereas under  $\text{Cu}^{2+}$  stress, the NaCl-induced reduction in oxidative damage only took place in root tissue.

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